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The crucial events leading to loss of cell viability after hypoxic and toxic injury remain poorly understood. Cell death is rarely synchronous, and rapidly occurring events leading to irreversible injury may be obscured when large cell populations are studied. To overcome this drawback, single cultured hepatocytes and myocytes during hypoxic and toxic stress were studied using a new, rapidly developing technology called Multiparameter Digitized Video Microscopy (MDVM). In MDVM, living cells are labeled with multiple fluorescent probes. Each probe responds to a defined cellular parameter by emitting fluorescence of a specific wavelength. 2-dimensional images of the fluorescence of each probe are digitized and stored during the course of cell injury. In this way, several cellular parameters are monitored quantitatively over time. Examples of parameters which we have observed with

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spatial and temporal resolution in individual living cells include cytosolic free Ca^{2+}, H^+ and Na^+, mitochondrial membrane potential, cell viability, lipid order (fluidity), and protein and non-protein thiols. Our major findings are: 1. Formation of plasma membrane blebs accompanies toxic and hypoxic injury. In hepatocytes, blebs initially are sites of increased membrane fluidity but later a transition to gel state occurs shortly before the onset of cell death. 2. Cell death happens abruptly and is preceded by a metastable period of increasing nonspecific permeability of the plasma membrane. Loss of viability often appears precipitated by rupture of a surface bleb. 3. An increase of cytosolic free Ca^{2+} is not a final common pathway leading to blebbing and cell death during hypoxic and oxidative stress. 4. A decrease of mitochondrial membrane potential precedes loss of cell viability and occurs early in some types of oxidative injury. 5. Cytosolic pH falls by more than 1 pH unit in parallel with ATP depletion and does not rise until a few minutes prior to the onset of cell death (metastable state). 6. Intracellular acidosis protects against cell killing during anoxic and toxic cell injury. Moreover, restoring acidotic pH to physiological levels greatly accelerates cell killing. This 'pH paradox' may to be an important factor in reperfusion injury to ischemic tissue. Our working hypothesis is that intracellular acidosis suppresses degradative processes (proteolysis, phospholipid hydrolysis, nucleic acid breakdown) activated by hypoxic and toxic stress. In the pH paradox, inhibition of these pH-dependent degradative processes is removed when acidotic pH is returned to normal, leading rapidly to cell death.

1. ROLE OF INTRACELLULAR pH IN THE 'pH PARADOX' OF REPERFUSION INJURY TO NEONATAL CARDIAC MYOCYTES (with John M. Bond and Brian Herman).

Myocardial ischemia is characterized by anoxia and a large decrease of pH. After a critical period of ischemia, reperfusion precipitates irreversible injury. Previous work from this laboratory showed that this reperfusion injury can be attributed in cultured neonatal myocytes to a rapid return to physiologic pH. The contribution of intracellular pH (pH_i) to this 'pH paradox' has not been defined. Accordingly, the aim of this study was to assess the role of pH_i in the pH paradox. Cardiac myocytes were isolated from 2-3 day neonatal rats and purified by centrifugal elutriation. After 5-9 days in culture, spontaneously beating myocytes were loaded with 7 μM BCECF/AM for 20 minutes at 37°C, mounted on a microscope stage, and incubated with 20 mM 2-deoxyglucose plus 2.5 mM NaCN (chemical hypoxia) in Krebs-Ringers-Hepes (KRH) at pH 6.2 to simulate the ATP depletion and acidosis of ischemia. pH_i dropped progressively from 7.5 to 6.5 over 1.5 hours and subsequently remained constant. After a total of 3 hours, the inhibitors were removed and the cells incubated in KRH at pH 7.4. After this reperfusion, pH_i increased. After 40 minutes and as pH_i approached 7, the myocytes blebbed, hypercontracted, and lost viability as assessed by propidium iodide exclusion. Conversely, if inhibitors were removed and the cells were incubated at pH 6.2 instead of 7.4, pH_i remained acidotic. Blebbing and hypercontraction were not observed, and no loss of viability was observed through 90 minutes. We conclude that a return of pH_i to a physiologic range is associated with cell death.

2. A NOVEL CYTOTOXICITY ASSAY TO MONITOR CELL VIABILITY CONTINUOUSLY IN CULTURED CELLS USING A MULTI-WELL FLUORESCENCE SCANNER: CYTOPROTECTION BY U74006F BUT NOT CALCIPHOR (with Anna-Liisa Nieminen, Roberto Imberti and Brian Herman).

Research on mechanisms of cell death is hindered by the lack of efficient methods to monitor continuously irreversible injury. Previously, we developed an assay to monitor cell viability non-destructively in cell suspensions (Am J Physiol 255,C315). Here, we describe a cytotoxicity screening assay suitable for cultured cells. Overnight cultured rat hepatocytes plated on 96-well microtiter plates ($1\text{-}5x10^4$/well) were incubated in Krebs-Ringer-Hepes buffer containing 10-50 μM propidium iodide (PI). As cells lost viability, their nuclei were labeled by PI with fluorescence enhancement measured with a multi-well scanner. Digitonin (375 μM) was used to permeabilize all cells and generate a fluorescence signal corresponding to 100% cell death. The increase of fluorescence correlated linearly with LDH release. Employing this technique, two reputed protective agents, U74006F and calciphor, were evaluated during 'chemical hypoxia' with KCN plus iodoacetate and oxidative stress with *t*-butyl hydroperoxide (*t*-BuOOH). U74006F (10-500 μM), an inhibitor of lipid peroxidation, protected against both chemical hypoxia and *t*-BuOOH. Calciphor, a mixture of prostaglandin B_1 oligomers, did not protect and was toxic at higher concentrations ($\geq$50 μM). In conclusion, a simple high capacity cytotoxicity screening assay was developed for cultured cells. U74006F but not calciphor protected against lethal injury in models of chemical hypoxia and oxidative stress.

3. ROLE OF pH IN HYPOXIC, ISCHEMIC AND REPERFUSION INJURY. (with John M. Bond, Robert C. Currin, Anna-Liisa Nieminen, Ronald G. Thurman*, and Brian Herman).

Hypoxia and acidosis are characteristic features of ischemia, tissue hypoperfusion and shock. Upon reperfusion, reoxygenation and a return to physiologic pH occur simultaneously. Much attention has been given to the role of reoxygenation and oxygen free radical formation in reperfusion injury, but little attention has been paid to the possible importance

of changes of pH. In isolated and cultured hepatocytes, cultured cardiac myocytes and isolated perfused livers, acidotic pH is highly protective against the onset of cell death during anoxia, during 'chemical hypoxia' with metabolic inhibitors, and after exposure to various toxic chemicals. Using multiparameter digitized video microscopy (MDVM) of BCECF fluorescence, it was demonstrated that this protection is mediated via intracellular acidification. Changes of pH and reoxygenation during ischemia and reperfusion were also modeled in cultured rat neonatal cardiac myocytes and isolated perfused rat livers by exposure to anoxia at pH 6.1-6.2 followed by reoxygenation at pH 7.4. Reperfusion in this model precipitates cell killing. Cell killing also occurs when pH is increased to 7.4 without reoxygenation but does not occur when cells are reoxygenated without a return to physiologic pH. In conclusion, acidotic pH protects against anoxic cell death, but a rapid return from acidotic to physiologic pH initiates the onset of cell death. This latter phenomenon, a 'pH paradox', may be an important factor contributing to reperfusion injury.

4. ROLE OF REOXYGENATION AND pH IN REPERFUSION INJURY TO CARDIAC MYOCYTES (with John M. Bond and Brian Herman).

Anoxia and a large decrease of pH are characteristic of myocardial ischemia. After a critical period of ischemia, reperfusion of myocardium results paradoxically in irreversible injury which has been termed a 'reperfusion paradox'. Accordingly, the aim of this study was to assess the relative importance of reoxygenation and changes of pH to reperfusion injury. Rat neonatal myocytes were purified by centrifugal elutriation and mounted in a gas-tight chamber on a microscope stage. Anoxia was created by infusion of 1 mg/ml submitochondrial particles (SMP) and 5 mM succinate. Respiration of SMPs depleted oxygen to less than 0.1 torr within 10-20 minutes. 2-Deoxyglucose (20 mM) was also added to inhibit glycolysis. Rates of cell killing were determined by propidium iodide labelling of cell nuclei. After 280 minutes, loss of myocyte viability was 92% at an extracellular pH of 7.4, 20% at pH 6.6, and 18% at pH 6.2. To model the changes of pH and oxygenation during ischemia and reperfusion, myocytes were subjected to anoxia at pH 6.2 for 240 minutes followed by reoxygenation at pH 7.4. Under these conditions, reperfusion precipitated 52% cell killing in 80 minutes (reperfusion paradox). When pH was increased to 7.4 without reoxygenation, cell killing was 33% (pH paradox), whereas when cells were reoxygenated at pH 6.2, no cell killing occurred (no oxygen paradox). We conclude that an acidotic extracellular pH protects against anoxic cell death. Moreover, we propose that a rapid return from acidotic to physiologic pH may be an important factor contributing to reperfusion injury -- a 'pH paradox'.

5. RECOVERY OF CULTURED RAT NEONATAL MYOCYTES FROM HYPERCONTRACTURE AFTER CHEMICAL HYPOXIA (with John M. Bond and Brian Herman).

Cardiac myocytes exposed to anoxia hypercontract into a blebbed rounded mass. Such hypercontraction is usually considered a manifestation of irreversible injury. Here, we studied functional recovery, long-term viability and ATP levels of cultured neonatal rat cardiac myocytes after metabolic inhibition with cyanide and 2-deoxyglucose, a model of 'chemical hypoxia' which mimics the ATP depletion and reductive stress of anoxia. After addition of inhibitors, 5-day cultured myocytes ceased spontaneous contractions within 1-2 minutes, blebbed and hypercontracted after 35 minutes, and lost viability after 100 minutes as assessed by nuclear labelling with propidium iodide. 11-day cultured myocytes exhibited a similar progression of injury. When the metabolic inhibitors were removed, spontaneous contractions resumed after an average of 11 minutes in non-hypercontracted myocytes and after 35 minutes in hypercontracted myocytes, regardless of the length of time spent in the hypercontracted state. Recovering cells remained viable and exhibited spontaneous contractions through 24 hours of observation, whereas contractility never returned in propidium

iodide-labelled cells. ATP levels decreased rapidly after chemical hypoxia and were partially restored upon washout of inhibitors. ATP recovery was similar in non-hypercontracted and hypercontracted cells although the time to recovery of spontaneous contractions was different. Thus, contractile dysfunction during recovery in hypercontracted cells was not due to lack of regeneration of ATP. In conclusion, contractile dysfunction following recovery from hypercontracture observed here may represent a cellular model for myocardial 'stunning'.

6. INTRACELLULAR DISTRIBUTION OF PROTEIN AND NON-PROTEIN THIOLS IN SINGLE CULTURED HEPATOCYTES BY MULTIPARAMETER DIGITIZED VIDEO MICROSCOPY (MDVM) (with Anna-Liisa Nieminen and Brian Herman).

Cellular glutathione plays an important role in protecting cells against damage from oxidant chemicals. The fluorescent probes monochlorobimane (mBCl) and monobromobimane (mBBr) have been used previously to measure intracellular glutathione and protein thiols by flow cytometry (J Biol Chem 263, 14107). Here, we use bimane probes to determine the intracellular distribution of thiols in single hepatocytes. Overnight cultured rat hepatocytes were stained with 200 μM mBCl or 500 μM mBBr. Fluorescence was imaged at 380 nm excitation and 470 nm emission using MDVM. Intracellular distribution of fluorescence was determined by opening intracellular compartments with increasing strengths of detergent. mBCl fluorescence which identifies non-protein thiols (predominantly glutathione) was 92% localized in cytosol. mBBr fluorescence released by 10 μM digitonin corresponded principally to glutathione, whereas the remainder corresponded to protein thiols. Protein and non-protein thiols were measured during chemical hypoxia with 2.5 mM KCN and 0.5 mM iodoacetate and during oxidative stress with 50 μM $HgCl_2$. $HgCl_2$ decreased protein thiols by 70% and glutathione by 95% in 90 seconds. In chemical hypoxia, glutathione was 50% depleted after 20 min but protein thiols declined more slowly. In conclusion, mBCl and mBBr are useful probes to measure protein and non-protein thiols simultaneously in single cells during injury.

7. MITOCHONDRIAL AND GLYCOLYTIC DYSFUNCTION IN HEPATOCYTES EXPOSED TO *t*-BUTYL HYDROPEROXIDE: CONTRIBUTION OF A MITOCHONDRIAL PERMEABILITY TRANSITION TO LETHAL INJURY (with Roberto Imberti, Anna-Liisa Nieminen, and Brian Herman).

In isolated mitochondria, *t*-butyl hydroperoxide (*t*-BuOOH) promotes a permeability transition characterized by increased permeability to small ions, swelling and loss of membrane potential. Cyclosporin A (CyA) and trifluoperazine (TFZ) inhibit this permeability transition. Here, we investigate the role of the mitochondrial permeability transition in lethal injury from *t*-BuOOH. Hepatocytes from fasted rats were isolated by collagenase perfusion, and cell viability was assessed by propidium iodide fluorescence. Mitochondrial membrane potential was evaluated by rhodamine 123 (Rh 123) retention. Glycolysis was determined from lactate plus pyruvate production. *t*-BuOOH caused dose and time-dependent cell killing. Fructose, a substrate for glycolytic ATP formation, protected at lower ($\leq$50 μM) but not at higher concentrations of *t*-BuOOH. At 100-300 μM *t*-BuOOH, oligomycin (10 μg/ml) conferred protection upon fructose. CyA (0.5 μM) plus TFZ (5 μM) could replace oligomycin in conferring protection. In single cultured hepatocytes, *t*-BuOOH caused abrupt leakage of Rh 123 from mitochondria after about 30 min which preceded cell death. In hepatocyte suspensions, CyA plus TFZ in the presence of fructose prevented mitochondrial depolarization. At 1 mM, *t*-BuOOH inhibited glycolysis. In conclusion, our findings support the hypothesis that inhibition of mitochondrial function is the common final pathway leading to cell death after exposure to *t*-BuOOH.

8. pH-DEPENDENT PHOSPHOLIPASE A_2 ACTIVITY CONTRIBUTES TO LOSS OF PLASMA MEMBRANE INTEGRITY DURING CHEMICAL HYPOXIA IN RAT HEPATOCYTES (with D. Corrine Harrison and Brian Herman).

Alterations in plasma membrane structure and function are known to accompany hypoxic and ischemic injury, yet the mechanisms responsible for the alterations and the role they play in lethal cell injury are not clear. Plasma membrane phospholipid composition represents a balance between phospholipid degradation and ATP-dependent reacylation. Since ATP-depleted cells cannot reacylate degraded phospholipids during hypoxic injury, we examined the potential role of plasma membrane phospholipid degradation in lethal cell injury. Cultured rat hepatocytes labeled overnight with 20 μCi/ml of ^{14}C arachidonic acid were exposed to KCN (2.5 mM) and iodoacetate (0.5 mM), conditions which mimic the ATP depletion and reductive stress of hypoxia. The rate of arachidonic release, used as a measure of phospholipase A_2 activity, was measured at 30 minute intervals at pH 7.4, in the presence or absence of the phospholipase inhibitors dibucaine (100 μM) or mepacrine (30 μM), or at pH 6.5. Cell viability was also determined under similar conditions using trypan blue staining. At physiological pH, in the absence of mepacrine or dibucaine, >90% of cells were dead by 60 minutes whereas only 30% of cells were dead at this same time point when the phospholipase inhibitors were present or the pH was lowered to 6.5. A similar difference in the rate of arachidonic acid release was also observed. Dibucaine, mepacrine and pH 6.5 all delayed the release of arachidonic acid relative to pH 7.4. The data indicate that activation of phospholipase A_2 activity occurs during hypoxic injury and that the activity is greater at neutral or slightly alkaline pH. Thus, acidosis may exert a protective effect on hypoxic injury by modulating the activity of phospholipase A_2.

9. CALCIUM-ACTIVATED PROTEOLYSIS CONTRIBUTES TO LETHAL OXIDATIVE INJURY IN ENDOTHELIAL CELLS (with M. Dominguez, M.-F. Ronveaux-Dupal and B. Herman).

Toxic metabolites of oxygen (oxygen free radicals (OFR)) from xanthine oxidase (XO) and other sources (neutrophils, arachidonic acid metabolism and mitochondria) may mediate ischemia-reperfusion injury to the endothelium of heart, lung, brain and kidney, and may also contribute to the development of atherosclerosis. OFR increase cytosolic free calcium (Ca^{2+}_i) in several cell types and an increase in Ca^{2+}_i through activation of Ca^{2+}-dependent degradative enzymes has been hypothesized to cause cell damage. Therefore, using Multiparameter Digitized Video Microscopy (MDVM), we measured Ca^{2+}_i in human umbilical vein endothelial cells during oxidative stress induced by xanthine (50 μM) plus xanthine oxidase (40 mU/ml) in the presence or absence of extracellular Ca^{2+} (Ca^{2+}_o) and protease inhibitors. Oxidative stress caused a large sustained increase in Ca^{2+}_i from a basal level of 150 nM to near 500 nM, which was preceded by formation of plasma membrane blebs. This increase in Ca^{2+}_i was dependent on Ca^{2+}_o, as removal of Ca^{2+}_o prevented the increase in Ca^{2+}_i during oxidative stress and prolonged cell viability. Readdition of Ca^{2+}_o resulted in an immediate large increase in Ca^{2+}_i and rapid onset of cell death. The protease inhibitors, leupeptin (10 μM) and pepstatin (10 μM) (in the presence of Ca^{2+}_o), delayed the increase in Ca^{2+}_i and prolonged cell viability. The results support the hypothesis that endothelial cell injury due to oxidative stress may be the result of damage to the endothelial cell plasma membrane allowing Ca^{2+} influx and activation of cellular proteases.

10. ASSESSMENT OF CELL DEATH IN HYPOXIC CELL INJURY (with Anna-Liisa Nieminen, Gregory J. Gores, Roberto Imberti, and Brian Herman).

The critical events which underlie the transition from reversible to irreversible injury remain unclear. Here, we review recent studies with rat hepatocytes where we have focused on lethal cell injury following anoxia and chemical hypoxia. The latter is a model of

metabolic inhibition with cyanide and iodoacetate which mimics the ATP depletion and reductive stress of anoxia. We observed three stages of cell injury during anoxia and chemical hypoxia. Stage I consisted of the formation of numerous bleb-like evaginations of plasma membrane. Subsequently in Stage II of injury, the blebs grew and coalesced by fusion to form one to a few large terminal blebs. Ultimately, one of the terminal blebs lysed causing fluorescent probes trapped in the cytosol to be lost and normally impermeant dyes such as propidium iodide to label cell nuclei. These changes mark the onset of cell death and the beginning of Stage III of injury. Because labeling of nuclei of non-viable cells by propidium iodide resulted in an enhancement of fluorescence, a new fluorometric assay using propidium iodide fluorescence was developed to monitor cell viability continuously and nondestructively in cell suspensions and cultured cells. Employing this cytotoxicity screening assay, protection by numerous agents against lethal injury was evaluated during chemical hypoxia. Acidosis was shown to protect strongly against the onset of cell death during ATP depletion from chemical hypoxia. The efficient glycolytic substrate, fructose, but not glucose also gave strong protection against cyanide toxicity provided that iodoacetate, a glycolytic inhibitor, was not present. Antioxidants and certain inhibitors of phospholipases and proteases protected implicating a role for oxygen radicals, phospholipases and proteases in lethal hypoxic injury. Osmotic agents which prevent cell swelling and miscellaneous other putative protective agents were without effect. The results show the utility of the propidium iodide cytotoxicity screening assay for characterizing hypoxic cell injury.

11. MITOCHONDRIAL DEPOLARIZATION DOES NOT LEAD TO INTRACELLULAR CALCIUM RELEASE IN CULTURED RAT HEPATOCYTES EXPOSED TO $HgCl_2$ OR UNCOUPLER (with Anna-Liisa Nieminen, Toru Kawanishi and Brian Herman).

Hg^{2+} binds with high affinity to protein and non-protein thiols. Using multiparameter digitized video microscopy (MDVM) of single cultured rat hepatocytes and the fluorescent probes monochlorobimane and monobromobimane, protein and non-protein thiol oxidation was virtually 100% complete after 90 seconds exposure to 50 μM $HgCl_2$. Within 1 minute following, mitochondria abruptly became depolarized as indicated by the redistribution of rhodamine 123 into the cytosol. No increase of cytosolic free Ca^{2+} measured by ratio imaging of Fura-2 fluorescence accompanied mitochondrial depolarization although free Ca^{2+} subsequently increased 3-4 minutes later. An identical sequence occurred in low Ca^{2+} (<2 μM) medium but without a late increase of Ca^{2+}. 2 μM CCCP, an uncoupler, was also added to depolarize mitochondria. No increase of free Ca^{2+} followed for several minutes in normal or ATP-depleted cells. In conclusion, no significant gradient of Ca^{2+} appears to exist between the cytosol and the mitochondrial matrix in cultured hepatocytes.

12. SUPPRESSION OF HORMONE AGONIST-INDUCED Ca^{2+} OSCILLATIONS IN CULTURED HEPATOCYTES BY CHEMICAL HYPOXIA (with Toru Kawanishi, Anna-Liisa Nieminen and Brian Herman).

The model of 'chemical hypoxia' with 2.5 mM KCN plus 0.5 mM iodoacetic acid (IAA) mimics the ATP depletion and reductive stress of anoxia. Here, we examined the effects of chemical hypoxia on hormone agonist-induced Ca^{2+} oscillations in 1-day cultured rat hepatocytes using Fura-2 ratio imaging and digitized video microscopy. 50 μM phenylephrine (Phe) and 20-40 nM Arg-vasopressin (Vas) induced Ca^{2+} oscillations in about 70% and 40% of cells, respectively. After KCN and IAA, both Phe and Vas-induced oscillations ceased after one spike. When KCN plus IAA were added 5 minutes prior to hormone agonist, no increase or oscillation of free Ca^{2+} occurred. Phe and Vas also increased cellular soluble inositol phosphates (IP_3 and others) by 22% and 147%, respectively, an effect which was suppressed by KCN plus IAA. Intracellular acidosis is characteristic of chemical hypoxia. Monensin (10 μM) prevented intracellular acidosis after KCN plus IAA but did not

prevent suppression of Ca^{2+} oscillations. Intracellular acidosis without ATP depletion was induced by addition of 40 mM Na acetate. This treatment also suppressed Ca^{2+} oscillations but did not inhibit hormone-induced inositol phosphate release. We conclude that ATP depletion during chemical hypoxia disrupts hormone-IP_3 coupling whereas intracellular acidosis may inhibit IP_3-induced Ca^{2+} release from intracellular stores.

13. PROTECTION BY ACIDOTIC pH AND FRUCTOSE AGAINST LETHAL INJURY TO RAT HEPATOCYTES FROM MITOCHONDRIAL INHIBITORS, IONOPHORES AND OXIDANT CHEMICALS (with Anna-Liisa Nieminen, Thomas L. Dawson, Gregory J. Gores, Toru Kawanishi and Brian Herman).

The importance of mitochondrial ATP formation and extracellular acidosis was evaluated in hepatocyte suspensions after different toxic treatments. Acidotic pH was protective against cell killing from all toxic treatments examined except for pronase, a toxic protease. Fructose, a substrate for glycolytic ATP formation, provided good protection against toxicity from cyanide, oligomycin, *t*-butyl hydroperoxide, menadione and cystamine. Protection by fructose against CCCP, gramicidin and Br-A23187 required oligomycin. This indicated that these ionophores were causing cytotoxicity by uncoupling oxidative phosphorylation. Fructose provided little protection against pronase and $HgCl_2$, the latter compound being a potent inhibitor of glycolysis. In conclusion, disruption of mitochondrial ATP formation was a common event contributing to the toxicity of chemical oxidants and ionophores. Acidotic pH was generally protective under these conditions of impaired ATP generation.

14. MITOCHONDRIA AS A SOURCE OF REACTIVE OXYGEN SPECIES DURING REDUCTIVE STRESS IN RAT HEPATOCYTES (with Thomas L. Dawson, Gregory J. Gores, Anna-Liisa Nieminen and Brian Herman).

In rat hepatocytes, loss of cell viability, oxygen consumption, and hydroperoxide formation were determined after metabolic inhibition with iodoacetate and different inhibitors of mitochondrial respiration ('chemical hypoxia'). Cell killing, hydroperoxide formation, and inhibitor-insensitive oxygen consumption were greater after azide than after myxothiazol or cyanide. Desferrioxamine, an inhibitor of iron-catalysed hydroxyl radical formation, delayed cell killing after mitochondrial inhibition, indicating that formation of reactive oxygen species was contributing to lethal cell injury. Myxothiazol and cyanide both inhibit Complex III (ubiquinol-cytochrome *c* oxidoreductase) of mitochondria whereas azide inhibits Complex IV (cytochrome *c* oxidase). Increased cell killing in the presence of azide indicates, therefore, that mitochondrial oxygen radical formation by Complex III can contribute cell killing during reductive stress.

15. LIPID ORDER IN HEPATOCYTE PLASMA MEMBRANE BLEBS DURING ATP DEPLETION MEASURED BY DIGITIZED VIDEO FLUORESCENCE POLARIZATION MICROSCOPY (with Kathyrn Florine-Casteel and Brian Herman).

Low-light digitized video fluorescence polarization microscopy was used to measure lipid order parameters in plasma membrane blebs of single, cultured rat hepatocytes during ATP depletion with the metabolic inhibitors cyanide and iodoacetic acid ('chemical hypoxia'). Hepatocytes were labeled on the microscope stage with the plasma membrane probe trimethylammonium diphenylhexatriene at successive stages of cell injury. A pair of fluorescence polarization ratio images was obtained from a series of four fluorescence images recorded with a polarizer in the emission path oriented first parallel and then perpendicular to each of two orthogonal excitation light polarization directions. From the polarization ratio images, the lipid order parameter, S, was determined in individual plasma membrane blebs. The results indicate that the plasma membrane becomes uniformly rigid

within a few minutes of the addition of metabolic inhibitors, when small surface blebs have formed and ATP levels have fallen by >95%. The measured order parameter of S~0.75 in normoxic cell plasma membranes, remained unchanged throughout the course of bleb development and ultimate cell death. These findings demonstrate that significant alteration in hepatocyte plasma membrane structure occurs early in hypoxic cell injury.

16. PROTECTION BY ACIDOTIC pH AGAINST ANOXIC CELL KILLING IN PERFUSED RAT LIVER: EVIDENCE FOR A 'PH PARADOX' (with Robert T. Currin, Gregory J. Gores and Ronald G. Thurman).

Reperfusion of ischemic tissues causes a paradoxical injury. Here, we measured lactate dehydrogenase (LDH) release as an indicator of tissue damage in perfused rat livers during anoxia and reoxygenation. During anoxia, LDH release was substantially reduced at acidotic pH (pH 6.1 to 6.9). Using anoxia at pH 6.1 followed by reoxygenation at pH 7.3 to model ischemia and reperfusion, an abrupt release of LDH occurred after reperfusion. A similar release of LDH occurred when pH of anoxic livers was increased to 7.3 without reoxygenation, but LDH release did not occur after reoxygenation at pH 6.1. Thus, a rapid increase of pH rather than reoxygenation accounted for tissue injury after reperfusion of ischemic liver.

17. CYTOSOLIC FREE MAGNESIUM, ATP, AND BLEBBING DURING CHEMICAL HYPOXIA IN CULTURED RAT HEPATOCYTES (with Andrew W. Harman, Anna-Liisa Nieminen and Brian Herman).

Cytosolic free Mg^{2+} concentration was determined in 1-day cultured rat hepatocytes using Multiparameter Digitized Video Microscopy (MDVM) of the fluorescent probe, mag-fura-2. Chemical hypoxia with KCN (5 mM) and iodoacetate (1 mM), a model which mimics the ATP depletion and reductive stress of hypoxia, caused a rapid increase of free Mg^{2+} from 1.1 ± 0.2 to 1.6 ± 0.2 mM within 4 min. Concurrently, numerous small plasma membrane blebs formed and ATP levels dropped from 13.2 to 1.3 nmol/10^6 cells. Removal of KCN and iodoacetate resulted in recovery of ATP to 60-70% of pre-exposure levels, a concomitant decrease in cytosolic free Mg^{2+} back towards basal levels, and reversal of blebbing (bleb resorption). These results indicate that changes of cytosolic free Mg^{2+} inversely reflect changes of ATP in a model of hypoxia and reoxygenation. Bleb formation and resorption were dependent on the fall and rise of ATP.

18. ASSESSMENT OF FURA-2 FOR MEASUREMENTS OF CYTOSOLIC FREE CALCIUM (with Michael W. Roe and Brian Herman).

Fura-2 has become the most popular fluorescent probe with which to monitor dynamic changes in cytosolic free calcium in intact living cells. We describe many of the currently recognized limitations to the use of Fura-2 in living cells and certain approaches which can circumvent some of these problems. many of these problems are cell type specific, and include: (a) incomplete hydrolysis of Fura-2 acetoxymethyl ester bonds by cytosolic esterases, and the potential presence of either esterase resistant methyl ester complexes on the Fura-2/AM molecular or other as yet unidentified contaminants in commercial preparations of Fura-2/AM; (b) sequestration of Fura-2 in non-cytoplasmic compartments (i.e. cytoplasmic organelles); (c) dye loss (either active or passive) from labeled cells; (d) quenching of Fura-2 fluorescence by heavy metals; (e) photobleaching and photochemical formation of fluorescent non-Ca^{2+} sensitive species; (f) shifts in the absorption and emission spectra, as well as the Kd for Ca^{2+} of Fura-2 as a function of either polarity, viscosity, ionic strength or temperature of the probe environment; and (g) accurate calibration of the Fura-2 signal inside cells. Solutions to these problems include: (a) labelling of cells with Fura-2 pentapotassium salt (by scrape loading, microinjection or ATP permeabilization) to circumvent the prob-

lems of ester hydrolysis; (b) labeling of cells at low temperature or after a 4°C pre-chill to prevent intracellular organelle sequestration; (c) performance of experiments at lower than physiological temperatures (i.e. 15-33°C) and use of ratio quantitation to remedy inaccuracies caused by dye leakage; (d) additional of N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) to chelate heavy metals; (e) use of low levels of excitation energy and high sensitivity detectors to minimize photobleaching or formation of fluorescent non-Ca^{2+} sensitive forms of Fura-2; and (f) the use of 340 nm and 365 nm (instead of 340 nm and 380 nm) for ratio imaging, which diminishes the potential contributions of artifacts of polarity, viscosity and ionic strength on calculated calcium concentrations, provides a measure of dye leakage from the cells, rate of Fura-2 photobleaching, and can be sued to perform *in situ* calibration of Fura-2 fluorescence in intact cells; however, use of this wavelength pair diminishes the dynamic range of the ratio and thus makes it more sensitive to noise involved in photon detection. Failure to consider these potential problems may result in erroneous estimates of cytosolic free calcium. By accurately assessing the contribution of each of these potential artifacts, it is possible to use Fura-2 to accurately estimate cytosolic free calcium in intact living cells.